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VITAL STAINING OF *COLIAS PHILODICE* AND *C. EURYTHEME*

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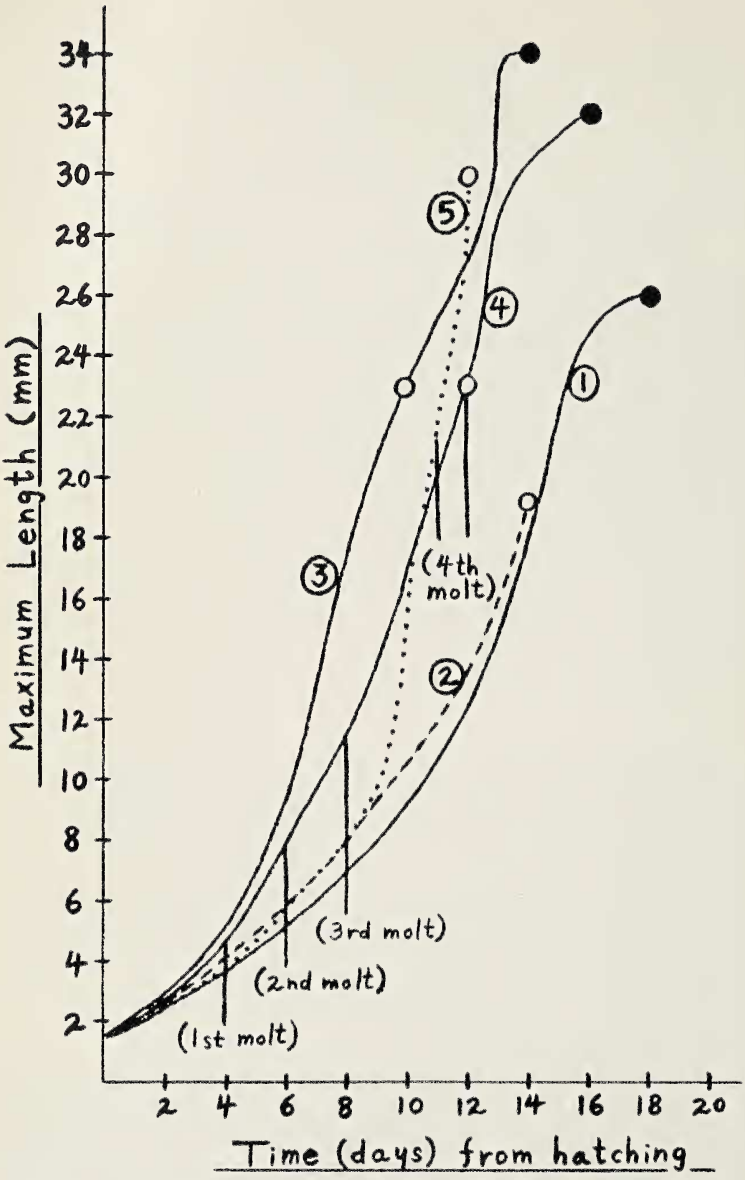
In experiments with the larvae of *Pieris rapae* (Linnaeus) it was found that ingestion of the dyes neutral red or Nile blue A imparted conspicuous external color (red or blue) to all three stages, while certain other dyes, notably brilliant cresyl blue, gave colored pupae but not adults (Kolyer, 1965). In the present work the three dyes mentioned were tried on the interrelated species *Colias philodice* (Latreille) and *C. eurytheme* (Boisduval) to observe the extent of manifestation of dye color in strongly pigmented butterflies (as opposed to the white *Pieris rapae*), to gain an indication of the generality of the vital staining method, and to optimize the procedure for dye-feeding in the case of the *Colias* species.

REARING

Oviposition. — In all cases the eggs were obtained by confining the female(s) in a screen-covered jar (approximately a cylinder of 3.5 inches inside diameter and 5 inches depth) with a layer of water in the bottom covered by a perforated wire screen through which sprigs of red clover (*Trifolium pratense*) were inserted. Oviposition was stimulated by direct sunlight or by electric light (suitably from a 100-watt bulb ten inches from the jar.) Oviposition began on zero day, and hatching started on the third day for broods 2 and 4 and the fourth day for the other broods (Tables 1 and 2).

Temperature and Humidity. — The broods were reared during July - September, 1962 for brood 1 and July - October, 1966 for broods 2-5. For broods 2-5 the breeding room reached a high of 94°F during the day and a low of 66°F at night, while the relative humidity was in the range 33-70% (usually 40-60%).


Maximum Length (mm)
34
32
30
28
26
24
22
20
18
16
14
12
10
8
6
4
2
2 4 6 8 10 12 14 16 18 20
Time (days) from hatching
(1st molt)
(2nd molt)
(3rd molt)
(4th molt)
1
2
3
4
5

Methods for Supplying Leaves. — Both broods (1 and 2) of *Colias eurytheme* were reared on cut leaves of red clover in cardboard boxes, e.g. shoe boxes as mentioned below, with gauze windows in the lids. The difficulty with this method was that rapid desiccation necessitated addition of fresh leaves up to five times a day. Cut leaves of white clover (*Trifolium repens*) were found to lose 50% of their original weight after 20 hours at 73-83°F and 34-45% relative humidity and 73% after 50 hours. To escape this difficulty, the following procedure was used for the three broods of *Colias philodice.* A cardboard shoe box, typically 10 inches long by 4 inches wide by 3.5 inches deep, with perforated bottom and a lid with gauze window, was mounted atop a wide-mouth jar (3.5 inches inside diameter) nearly filled with water, into which white clover stems were inserted. Since the young larvae tended to drop from the leaves when disturbed, the clover leaves at first were situated at some distance from one large perforation by bending over a bundle of stems and taping them to the floor of the box. Later, when the larvae were in the fourth instar, the whole area of the box above the jar was perforated with small holes, and individual stems were inserted to give a "carpet" of clover leaves.

Figure 1 shows that the *philodice* larvae fed by the stems-in-water method developed more rapidly and attained larger size than the *eurytheme* larvae fed cut leaves. Klots (1951) lists a greater maximum expanse for *eurytheme* (2.4 inches) than for *philodice* (1.9 inches), and the forewing length for females collected in the general vicinity of Morristown, New Jersey, was greater for *eurytheme* (29 mm, mean of seven specimens) than for *philodice* (26 mm, also mean of seven specimens). Also, the larva of *eurytheme* has been described as attaining a slightly greater length than that of *philodice* (Comstock and Comstock,

Fig. 1. Growth curves for larvae of broods 1-5.

The curves are identified by brood no.; see Tables 1 and 2. The open circles mark the beginning of dye-feeding. In the case of broods 3 and 4 some readings were taken beyond this point, but these are not equivalent to the preceding values and are plotted only to show the greater length attained and more rapid development of these broods (vs. 1 and 2) even with the retarding effect of dyes.

The solid circles mark the beginning of pupation.

Maximum larval length was noted daily and plotted vs. time, and smooth curves were drawn through the points. In actuality there are short no-growth steps, especially preceding the fourth molt. Approximate positions of the molts are indicated on the graph for brood 4 (and the fourth molt of brood 5).

As a weight reference, two 25 mm larvae from brood 1 weighed 0.180 and 0.213 grams.

Table 1

CONDITIONS FOR DYE-FEEDING

Brood No.	Colias Species	Dye (% of blend)	Feeding Start Larval Instar	Feeding Start Time, day	Time Dye Fed, days	Pupation, day	Yield Pupae	Color Pupae
1	eurytheme	none	-	-	-	23-31	(34 from 65 1st-instar larvae)	green
2	eurytheme	Nile blue A (5)	22-24	20-21	1.5-1.8	22-27	10/10	one blue (15 mm), rest sl. blue-green at most
		neutral red (5)	19-24	17-21[1]	5	23-26	7/9	pink (esp. on abdomen) to deep red
		Nile blue A (1) and neut. red (1)	22	21[1]	3-5	24-26	5/5	green to green with blue abdomen
		brill. cresyl blue (5)	18-20	21[1]	4-5	25-26	3/3	green
3	philodice	Nile blue A (5)	15-23	14	0.8-1.4	18-19	4/20	blue-green[2]
		Nile blue A (1)	24-27	16	2.3-4.5	20-22	2/8	greenish-blue; blue
	(1)	neutral red (5)	15-19	14[1]	4-5	18-19	7/10	dark-red
	(2)	neutral red (5)	26-30	18	0.5	20-21	3/3	two dark-red; one green with red abdomen
		Nile blue A (1) and neut. red (1)	17-19	15	0.5	19	4/5	sl. blue tint
		brill. cresyl blue (5)	18-19	15[1]	3-4	18-19	4/4	sl. grayish green
4	philodice	neutral red (5)	28-32 (one at 24)	16-19[1]	0-3	19-21	32/32	red to dark-red
5	philodice	Nile blue A (1)	27-30	16-17[1]	2-3	18-20	4/10	blue-green

[1]red dye to pupation. [2]green for controls.

1943). Therefore, it seems reasonable that *philodice* is not inherently larger than *eurytheme* but, if anything, smaller, and that the improved rearing procedure was responsible for the more rapid growth rates and greater ultimate lengths of the *philodice* larvae as well as the greater expanse of the adults (See forewing measurements in Table 2).

Mortality seemed reduced by the improved feeding method; for example, in brood 4, 39 eggs yielded 34 larvae which yielded 32 adults, while in brood 1 (cut leaves) 65 first-instar larvae (from approximately 150 eggs) yielded only 34 pupae, and in brood 2 (also cut leaves) only 34 larvae remained of the original 54 (from about 111 eggs) when the largest had reached 19 mm. No crowding problem was evident in brood 4 when the newly-hatched larvae (34) were reared to give 33 fifth-instar larvae (29 mm maximum length) in a single box of dimensions specified above.

Dye-Feeding Technique. — Dyes were fed by coating the leaves on both sides with dye-mica blend prepared by grinding and tumbling the dye with P-12 Davenite mica (325 mesh; Hayden Mica Co., Wilmington, Mass.) as described in the earlier paper (Kolyer, 1965). However, the dye/leaf ratio in the present work was higher than with *Pieris rapae* because more dye blend was used (about 5 mg for a white clover leaf 1.5 cm wide) and because clover leaves are far less thick than the average cabbage leaf.

The dyes (biological stains), all from National Aniline Division, Allied Chemical Corp., New York City, were brilliant cresyl blue (Colour Index No. 51010), neutral red (chloride, 70% minimum strength, 1.0% maximum water-insoluble content, Colour Index No. 50040), and Nile blue A (sulfate, 70% minimum strength, Colour Index No. 51180).

Explanation of Table 1. — The days listed for inception of dye-feeding, pupation, and eclosion in the tables are all based on the start of oviposition, which continued no more than three days, at zero day. In the Yield Pupae column, 7/9, for example, means that 7 pupae were obtained from 9 larvae fed dye. The pupal color was noted when pupae were about two days old.

Description of Female Parents. —

Brood 1. — Eggs (approximately 150) were laid by one *eurytheme,* moderately marked with orange, forewing 23 mm, taken near Fall River, Mass., on July 22, 1962.

Brood 2. — Eggs (approximately 111) were laid by some or all of five *eurytheme,* most by one moderately marked with orange,

Table 2

DATA ON ADULTS

Brood No.	Dye (% of blend)	Eclosion, day	Color Pupal Case	No. of Adults male	No. of Adults female	Forewing Length, mm, mean (range)	Ground Color	Eye Color	Color Intersegmental Abdominal Membrane
1	none	29-38	pale yellow	15	14	male-21(20-23) female-22(20-24)	orange	lt. green	lt. green
2	Nile blue A (5)	28-33	pale blue	6	4	male-19(15-22) female-23(21-24)	orange[1]	deep green	blue
	neut. red (5)	28-32	pink	5[2]	0	male-18(15-21) deep orange for unexpanded specimens	orange; golden-brown		pink
	Nile blue A (1) and neut. red (1)	30-33	pale blue	1	4	male-18 female-20(18-23)	orange	lt. green	blue
	brill. cresyl blue (5)	31-32	pale yellow	1[3]	1	male-20 female-20	orange	lt. green	lt. green
	none (control)	32-33	pale yellow	3	2	male-22(21-24) female-24(23,24)	orange	lt. green	lt. green
3	Nile blue A (5)	23-25	pale greenish-yellow	2	2	male-23(22,23) female-27(26,28)	faint green tint (1 male)	green	blue
	Nile blue A (1)	26-29	pale greenish-yellow	1	1	male-21 female-21	faint green tint in male	green	blue
	(1) neut. red (5)	23	pink	1[4]	0	-	orange	red	deep-red
	(2) neut. red (5)	25-27	flesh-color	1	2	male-23 female-26(25,26)	sl. deeper golden-yellow to orange-tan	yellow to deep-red	red
	Nile blue A (1) and neut. red (1)	24-25	pale yellow	0	4	female-27(26-29)	yellow	lt. green to green	blue
	brill. cresyl blue (5)	23-25	pale greenish-yellow	1	3	male-20 female-22(21-23)	yellow	lt. green	gray
	none (control)	24-25	pale yellow	0	3	female-29(28-29)	yellow	lt. green	gray
4	neut. red (5)	25-29	flesh-color	12	9Y[5] 11A[5]	male-23(20-25) female-24(23-26)	Y-orange-tan A-pink	Y-golden to deep-red A-deep-red	red
5	Nile blue A (1)	24-25	pale blue or greenish-blue	2	1	male-21(21,21) female-21	yellow-green (males), pale blue (albino female)	green	blue

[1] one sl. greenish inside border; see Plate 1. [2] two failed to expand.
[3] another eclosed but not expanded. [4] not expanded. [5] Y means yellow; A means albino.

forewing 26 mm, taken at Berkshire Valley, New Jersey, on July 3, 1966.

Brood 3. — Eggs (81) were laid by three *philodice,* wings yellow with no trace of orange, forewings 24, 26, 27 mm (mean 26 mm) taken at Morristown, New Jersey, on July 12 and 13, 1966.

Brood 4. — Eggs (39) were laid by three albinos, presumably *philodice* (as judged by width of the black borders of the forewings) with no trace of yellow or orange in the ground color, forewings 24,25,26 mm (mean 25mm), taken at Berkshire Valley, New Jersey, on September 3, 1966.

Brood 5. — Eggs (11) were laid by an albino female with a touch of yellow-orange in the wings, forewing 25 mm, and/or a yellow *philodice,* no trace of orange, forewing 27 mm, both taken at Morristown, New Jersey, on September 10, 1966.

RESULTS

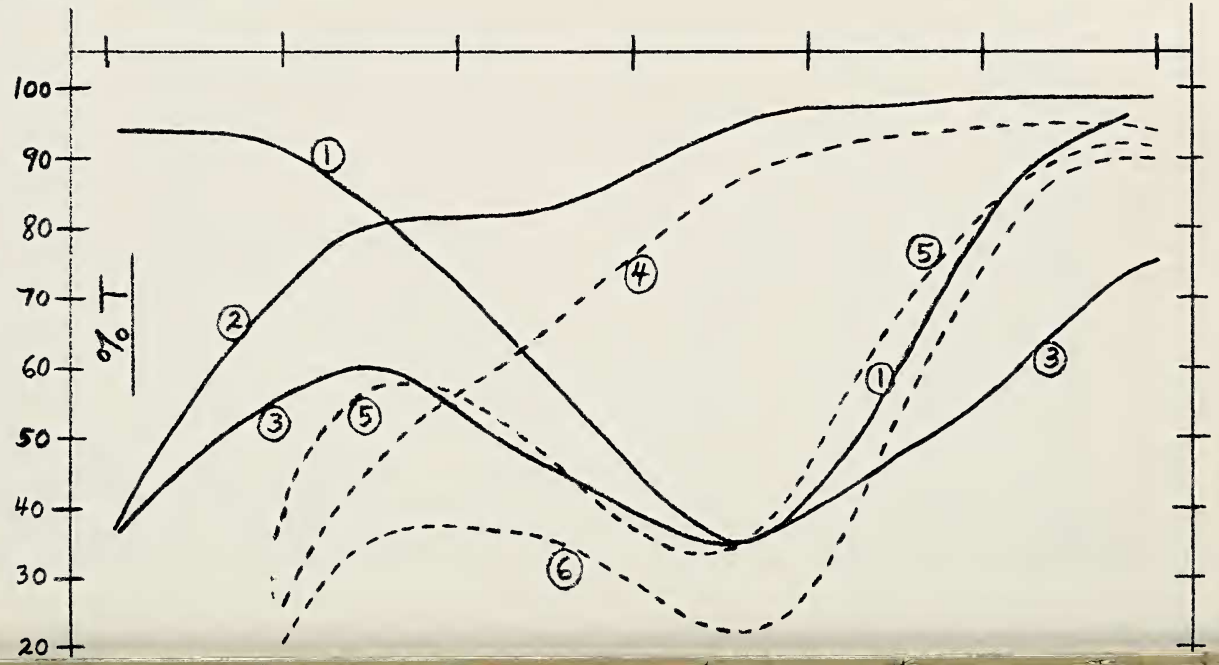
Color of Adults. — The situation is complicated by the fact that *philodice* and *eurytheme* are peculiarly interrelated and hybridize to some extent; see, for example, Gerould (1946) and Hovanitz (1949). However, the adults reared in broods 1 and 2 were typical *eurytheme,* well marked with orange, while the undyed adults reared in brood 3 were all typical *philodice,* like the parent females, with no trace of orange. In brood 5 the female was an albino, and the males appeared to be typical *philodice.* Only in brood 4 among the *philodice* broods was there a trace of *eurytheme;* a male and female which pupated without feeding on the dye (neutral red) showed a faint orange suffusion in the basal area of the forewing, and this could be observed in some of the dyed specimens (except the albinos, which were evenly pink like dyed *Pieris rapae* and presumably would have been quite uncolored if not fed neutral red). The introduction of color solely by neutral red as opposed to *eurytheme* genes seems unequivocal in brood 3, in which all 17 adults except the four fed neutral red (e.g., no .6, Plate 2) were quite yellow (with very faint greenish tint in some of the six fed Nile blue A). Approximately half the females in brood 4 were albinos, which form is known to be controlled by a dominant allele (for example, Hovanitz, 1944).

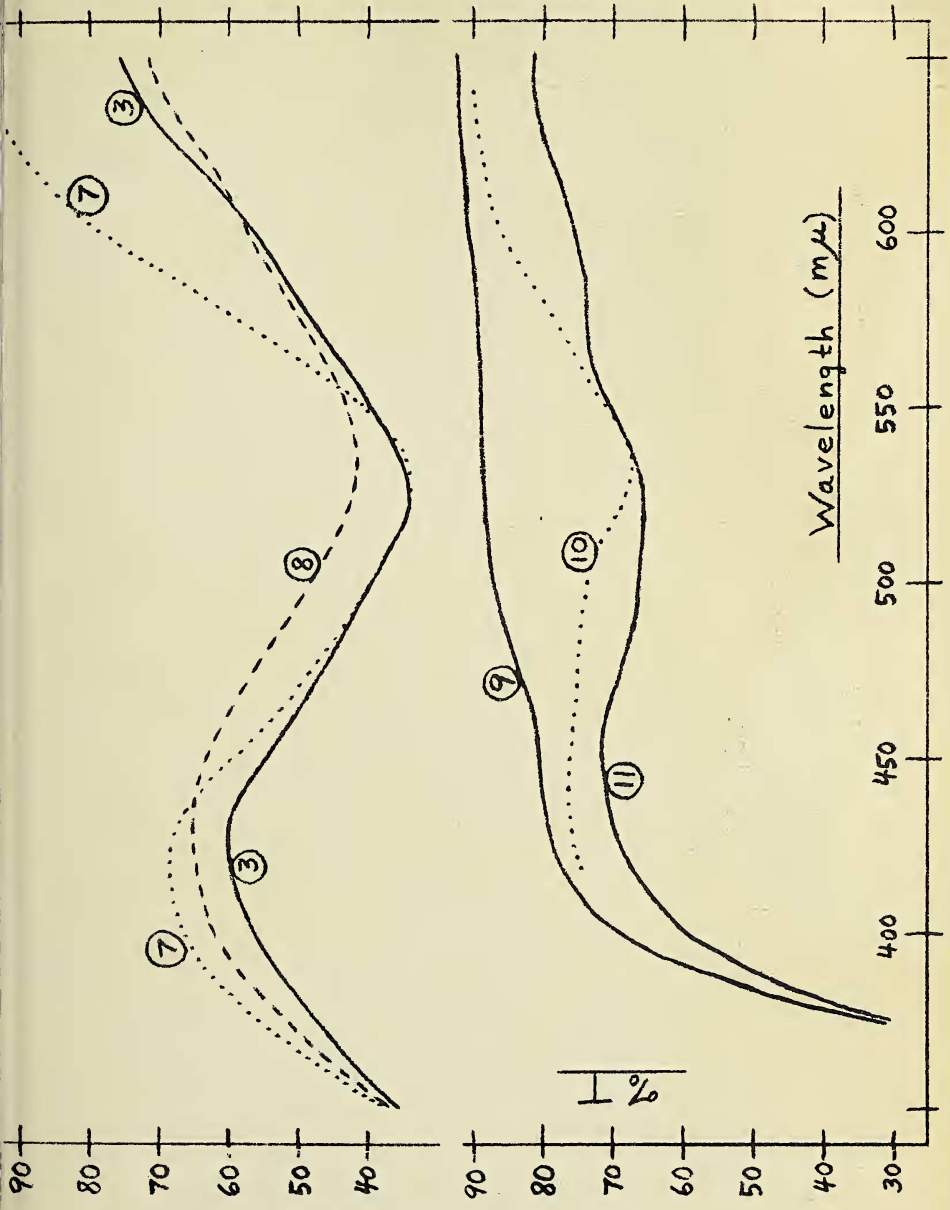
The color of the antenna club was altered by dye-feeding. This was tan in the controls (either species) and in specimens fed brilliant cresyl blue but was deep-green when Nile blue A had been fed and red-brown when neutral red had been fed (orange

Fig. 2. Spectra of extracts of adults stained with neutral red.

All spectra are for extracts of **Colias philodice** specimens. Spectra 4-6 were run at greater sensitivity than 1,2,3,7, and 8, and 9-11 were run at still greater sensitivity. % T = percent transmittance.

1 — neutral red (12 mg/1) in 1.20 ml concentrated hydrochloric acid (37-38% HCl) plus 3.75 ml concentrated ammonium hydroxide (28-30% ammonia); **2** — extract of body (36.5 mg) of albino female; see text for extraction procedure; **3** — extract of body (26.6 mg) of pink albino female fed neutral red (brood 4; resembled no. 2, Plate 1). **4** — extract (boiling water) of whole albino female (46.9 mg); see text for procedure. **5** — extract (boiling water) of whole pink albino female (39.1 mg) fed neutral red (brood 4). **6** — extract (boiling water) of whole albino female (spectrum 4) with neutral red added at 0.028 mg/7.0 ml extract. **7** — neutral red spectrum (1) and albino body spectrum (2) combined by adding optical densities. **8** — extract of albino female body (spectrum 2) with neutral red added at 0.055 mg/4.95 ml extract. **9** — extract of albino female forewing; see text for procedure. **10** — neutral red spectrum (at appropriate concentration) and albino forewing spectrum (9) combined by adding optical densities. **11** — extract of forewing (2.0 mg) from pink albino female fed neutral red (brood 4).




Wavelength (mμ)
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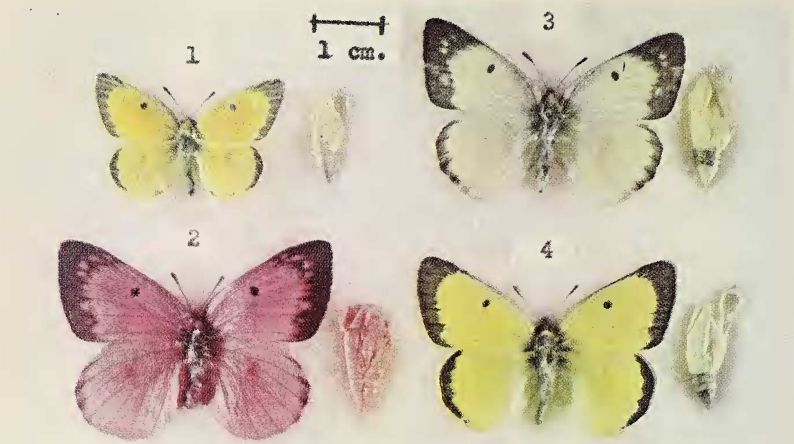

PLATE 1. SPECIMENS STAINED WITH NILE BLUE A AND NEUTRAL RED BY FEEDING DYES TO THE LARVAE.

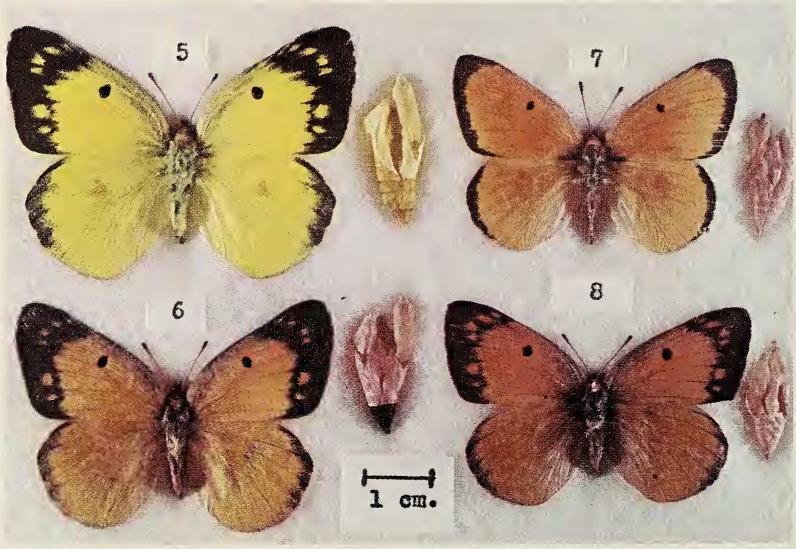

PLATE 2. SPECIMENS STAINED WITH NEUTRAL RED BY FEEDING THE DYE TO THE LARVAE.

1 — male **Colias eurytheme,** forewing 15 mm, fed Nile blue A, brood 2, from blue pupa (length 15 mm vs. normal length of 20 mm); **2** — albino female **C. philodice** fed neutral red, brood 4. **3** — albino female **C. philodice** fed Nile blue A, brood 5. **4** — male **C. philodice** fed Nile blue A, brood 5. **5** — female **C. philodice** control, brood 3. **6** — female **C. philodice** fed neutral red, brood 3. **7** — male **C. philodice** fed neutral red, brood 4. **8** — female **C. philodice** fed neutral red, brood 4.

The pupal case is shown with each specimen.

in the case of albino females).

The liquid voided following eclosion was blue-green for adults fed Nile blue A and strongly red for those fed neutral red.

Verbal description of color is vague at best (and photographic reproduction is hardly accurate), but the direction of vital staining should be evident from Table 2 as well as Plates 1 and 2.

Explanation of Table 2. — Forewing length is the distance from the base to the apex of the wing. The eye color was noted on living adults with the aid of a 16-power stereo microscope. The color of the abdominal membrane was observed by flexing the abdomen of a living specimen under the microscope to separate the segments. This was a sensitive indicator of the presence of Nile blue A or neutral red.

Concentration of Neutral Red in Adults. —

Paper Chromatography on Wings. — Single forewings were extracted by grinding the wing in a mortar with 1.0 ml concentrated hydrochloric acid (37-38% HCl). The liquid and undissolved debris were transferred with about three ml distilled water to a watchglass and evaporated to dryness on a hot plate. The soluble part of the residue was taken up in 0.15 ml distilled water and chromatographed by the ascending method (for a general description see Lederer and Lederer, 1953). Whatman No. 1 filter paper (3 inches by 4.5 inches) was made hydrophobic, in the manner of Ciglar, Kolsek, and Perpar, 1962, by dipping in 10% lauryl alcohol in 95% ethanol and allowing to dry. Spots (about 3 mm diameter) were applied with a capillary 5/8 inch from the shorter edge of the paper; three applications were made, with drying between, to reinforce each spot. The paper was dipped, spotted edge down, to a depth of 1/4 inch in a layer of solvent (2 volumes 95% ethanol : 2 volumes concentrated ammonium hydroxide (28-30% ammonia) : 1 volume distilled water) in a closed jar and left for one hour at room temperature (27°C). After drying in a draft of air the chromatogram was sprayed with 1.5% aqueous HCl to bring out the pink spot for neutral red.

For a female *philodice* fed neutral red (brood 4, resembling no. 8, Plate 2) the tan spot, Rf 0.75, due to wing pigments, was well separated from the pink spot, Rf about 0.24, for neutral red; wings of undyed *philodice* or *eurytheme* gave only the tan spot, as expected. Several quantities of neutral red (from same sample used to prepare the blend with mica) were evaporated with concentrated hydrochloric acid and chromatographed in the same way to allow a semiquantitative estimation of neutral red level in the wing by comparison of spot intensities. The result was very

approximately 0.01 mg neutral red, or, since the wing weighed 2.0 mg., 0.5% neutral red in the wing. The dye seems confined to the scales, incidentally; the membrane is colorless.

Colorimetry. — A Bausch and Lomb Spectronic 20 Colorimeter (band pass 20 millimicrons) was used to take % transmittance readings (readily converted to optical density, which equals the negative logarithm, to the base 10, of the transmittance) at every 10 millimicrons (in regions of absorption maxima) in the range 350-650 millimicrons (3500-6500 Angstoms). The % transmittance was readjusted to 100 with solvent at each wavelength. The sensitivity setting of the instrument was varied from nearly a maximum level for the wing extracts to lower levels for the other determinations. The spectra within each of the three sets were run at the same sensitivity and included standard neutral red solutions for calibration.

Body. — The body of a dried specimen (wings removed) was ground in a mortar with 1.20 ml concentrated hydrochloric acid (37-38% HCl), and the mixture was partly neutralized (to pH about 0.3) by adding 3.75 ml ammonia solution prepared by diluting one volume concentrated ammonium hydroxide (28-38% ammonia) to five volumes with distilled water. Debris was removed by filtration. The spectra of dyed and undyed albino bodies (nos. 2 and 3) are shown in Figure 2 as well as the spectrum of neutral red in the same solvent. The absorption maximum for neutral red was 530 millimicrons, as has been reported for acid solutions (Meyer and Treadwell, 1952), and a calibration curve (optical density vs. concentration) was obtained for this wavelength using several neutral red concentrations. By summing the optical densities of spectra 1 and 2, the predicted spectrum (neutral red superimposed on the undyed albino body extract) was obtained (no. 7). This was a fair match for the actual spectrum of the dyed albino (no. 3) except for considerable deviation above 550 millimicrons. However, addition of the amount of neutral red (as a concentrated solution) predicted for the dyed albino body by means of the calibration curve to the undyed albino body solution gave spectrum 8, which is in rather good agreement with the spectrum of the dyed albino body over the whole wavelength range. Since the level of neutral red in the extract of the dyed albino body was 0.066 mg (after correcting for the difference between spectra 8 and 3) and the body weighed 26.6 mg, the neutral red extracted amounted to 0.25% of the body. This is about double the level for the living body, since this species should lose about half its original weight on

drying (Kolyer, 1963).

Whole Adult. — The whole (dried) butterfly was ground in a mortar with about 5 ml acetone, transferred to a flask with about 25 ml acetone, and refluxed for 20 minutes with the intention of degreasing the body. Then the acetone was evaporated and replaced by about 10 ml distilled water. After refluxing for 30 minutes (neutral red is not affected by this treatment), the mixture was evaporated to about 4 ml, 0.035 ml concentrated hydrochloric acid was added, and the mixture was diluted to 7.0 ml with distilled water and filtered to remove debris. In Figure 2, spectra 4 and 5, respectively for undyed and dyed albinos, are generally similar to spectra 2 and 3 for the bodies. Addition of neutral red to the undyed albino extract gave spectrum 6, and the calibration curve indicated 0.022 mg in the extract or 0.06% neutral red in the butterfly. A dyed (orange) female (brood 4, resembling no. 8, Plate 2) and an undyed yellow female gave rather similar spectra to those for the albinos, and the neutral red level was the same (0.06% of the dyed butterfly, which weighed 37.0 mg). The low results suggest that this extraction method was less effective than trituration with concentrated hydrochloric acid, which dissolves much of the specimen.

Wing. — Single forewings were extracted in the same manner as the bodies (above). As in the case of the bodies, the calculated spectrum for the dyed albino (no. 10 in Figure 2) deviated from the observed spectrum (no. 11) above 550 millimicrons. By subtracting the optical density of spectrum 9 (undyed albino) at 530 millimicrons from than of spectrum 11 and converting to neutral red concentration by means of a calibration curve, a value of 0.0036 mg was found for the forewing of the dyed albino; this is 0.18% of the wing, which weighed 2.0 mg. Similarly, 0.0038 mg neutral red (0.19% of the wing) was found for the forewing of a dyed (orange) female from brood 4 resembling no. 8 in Plate 2. Incidentally, the spectrum of the extract of an undyed, yellow *philodice* wing showed 24% transmittance at 400 millimicrons and rose to a plateau of about 79% by 570 millimicrons, while an undyed *eurytheme* wing was less transparent in the lower (blue) region (12% transmittance at 400 millimicrons) and more transparent (e.g. 90% transmittance at 590 millimicrons) in the yellow and red regions. This is consistent with the presence of orange pigment.

Concentration of Nile Blue A in Adults. — No assay was made on the three *philodice* specimens conspicuously colored by Nile blue A (brood 5). However, the intensity of this dye is com-

parable to that of neutral red; for solutions of equal weight concentration neutral red gave optical density 0.27 at 530 millimicrons vs. 0.41 for Nile blue A at its maximum of about 640 millimicrons (Merrill and Spencer, 1948, report 634 millimicrons). Therefore, visual estimation suggests a concentration of on the order of 0.1% Nile blue A in the wings of the dyed albino from brood 5 (no. 3 in Plate 1).

DISCUSSION

Effect of Dyes on the Colias species vs. Pieris rapae. — The toxicity of Nile blue A was more pronounced with the *Colias* species than *Pieris rapae* when the level ingested was high enough to alter the wing color conspicuously. In brood 5, three well-dyed specimens were obtained (two shown in Plate 2) only at the expense of 70% mortality. As with *Pieris rapae,* Nile Blue A preferentially showed itself in *Colias* adults (abdominal membrane) when fed with neutral red at equal levels (Table 2).

Neutral red gave strongly pink specimens with 30% mortality with *Pieris rapae* (Kolyer, 1965), and good coloration with no mortality was achieved with *Colias philodice* (brood 4) when feeding was begun at the proper larval length.

Brilliant cresyl blue gave strongly violet pupae when fed to *Pieris rapae* larvae for 3-9 days, but when fed (at a higher dye/food ratio) to *Colias philodice* for 3-4 days (brood 3) there was only a subtle change in pupal color (Table 1). A feeding period of 4-5 days had no noticeable effect on the color of *C. eurytheme* pupae (brood 2). It is interesting that this dye gave very different results for *Pieris* vs. *Colias.*

Toxicity and Growth-Retarding Effect of Dyes. — Brilliant cresyl blue may have shown a slight retarding effect on the *Colias* species, while Nile blue A was toxic to the point of being very "tricky" to utilize and, besides killing larvae, produced some undersized specimens, the smallest being the dwarfed male shown in Plate 1.

Neutral red retarded growth and had a toxic effect in *C. eurytheme* when feeding was begun too early; a dwarfed male with 15 mm forewing was produced in brood 2, and only three properly-expanded adults were obtained from nine larvae. In this case the ground color was noticeably changed only in the unexpanded specimens, presumably because it was difficult for neutral red to manifest itself against the orange pigment. With *C. philodice,* larval growth was retarded (maximum length attained was 27 mm) and undersized, deformed pupae resulted

when neutral red feeding was begun too soon (group(1) in brood 3). However, when feeding was begun (brood 4) with larvae that had attained 83-94% of the ultimate length for controls (34 mm), there were no deaths, and only about five specimens out of 31 were not well tinged with pink. A single specimen (brood 4) fed neutral red from a length of 24 mm (71% of ultimate length) eclosed but failed to expand. It should be noted that staining with neutral red is reversible with *Colias* as with *Pieris rapae* and the silkworm (Edwards, 1921); loss of dye begins when the larva is transferred to undyed leaves. This effect is avoided, of course, by feeding the dye to pupation.

Optimum Dye-Feeding Procedure. — In agreement with the cited work with *Pieris rapae,* neutral red is judged particularly suitable for vital staining of the *Colias* species. The best procedure (at the level used in the experiments) is to confine dye-feeding to the latter part of the final instar. Alternatively, it is possible that the dye might be fed earlier if used at a lower concentration; a brief experiment with 1% neutral red in the blend showed that the larvae were visibly stained (as indicated conveniently by change in color of the lateral stripe) after being fed dye for 12 hours.

CONCLUSION

Neutral red has been found to be an effective vital stain, with an acceptable degree of toxicity, in *Pieris brassicae* (linnaeus) (Roer, 1959), *Pieris rapae* (Kolyer, 1965), the *Colias* species (present paper), and also the silkworm (Edwards, 1921). Vital staining provides indelible marking, which might be of use in the rearing of experimental broods, but an area of more interest might be the effect of the unusual color on the behavior, e.g. mating, of adult butterflies. The concentration of dye in the adult eye might alter response to light, for example; this has been suggested as an area for experimentation with moths fed rhodamine B (Vail, Howland, and Henneberry, 1966).

SUMMARY

Nile blue A and neutral red can be used to stain living larvae, pupae, and adults of *Colias philodice* and *eurytheme* by feeding leaves coated with dye extended with powdered mica. The usefulness of Nile blue A is limited by its toxicity, but neutral red is effective with little or no mortality when fed to larvae within

two or three days of pupation. The wings of yellow *Colias philodice* adults were stained by neutral red to a dull orange color, while dyed albinos were strongly pink. Paper chromatography and colorimetry on extracts of dried specimens indicated a level of on the order of 0.2-0.5% neutral red in the wing and 0.3% in the dried body. Brilliant cresyl blue, which is effective in staining the pupa of Pieris rapae, had little efficacy with the *Colias* species.

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